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Effect of percolation and chemical form on Pb bioavailability and toxicity to the soil invertebrate *Enchytraeus crypticus* in freshly spiked and aged soils☆

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ABSTRACT

In standard terrestrial ecotoxicological tests, soils usually are spiked with highly soluble metal salts leading to overestimation of bioavailability and introducing counterions that may contribute to toxicity. Leaching is suggested as an efficient method to avoid the effects of the associated counterions. The present study aimed at investigating the effects of leaching on the bioavailability and toxicity of $Pb(NO_3)_2$ and PbO to the potworm *Enchytraeus crypticus* in LUFA 2.2 soil freshly spiked or after 18 months ageing. Percolation decreased porewater Pb concentrations as well as the toxicity of both Pb forms. The influence of percolation differed between the two Pb forms and between freshly spiked and aged soils. Percolation slightly increased LC50s based on total soil Pb concentrations for $Pb(NO_3)_2$, but not for PbO, and only affected Pb toxicity to enchytraeid reproduction in freshly spiked soils. The differences in Pb uptake in *E. crypticus* and toxicity between the two Pb forms as well as between different treatments could be minimized by relating them to 0.01 M $CaCl_2$-extractable concentrations. In addition, body Pb concentrations could well explain enchytraeid survival across all soils and treatments, indicating its suitability as a good proxy for Pb toxicity in soil.

1. Introduction

Among soil contaminants, metals have received much attention due to their persistency. For instance, as a naturally occurring element, lead (Pb) is still a major contaminant in soils along highways even several years after the ban on lead-based additives in gasoline (Santorufo et al., 2012). It is a common metal contaminant in residential and industrial areas, resulting from anthropogenic activities (e.g. agriculture, industry, waste disposal and the use of leaded products). Total Pb concentrations at polluted sites may be as high as >16,000 mg Pb/kg dry soil, by far exceeding the limits set in regulatory standards (e.g. 100 mg/kg for lead in the soil according to the World Health Organization (WHO)), and contributing to strong detrimental effects on soil-dwelling organisms and human health via direct contact or food chain transfer (Alloway, 2013).

Striking differences in metal toxicity were observed between laboratory-spiked and historically contaminated soils (Lock et al., 2006; Smolders et al., 2015). In laboratory ecotoxicity tests investigating the effects of metals on soil organisms, soils usually are spiked with aqueous solutions of highly soluble metal forms, like $Pb(NO_3)_2$, $PbCl_2$, and Pb acetate (Bur et al., 2012; Darling and Thomas, 2005; Langdon et al., 2005; Zhang and Van Gestel, 2017). These forms, however, are not representative of the form in which Pb may end up in the soil and cannot mimic the realistic situation in the field due to the lack of a process called “ageing”. This ageing process (including complexation with organic and inorganic components of the soil and soil solution, sorption to the surface of clay particles, exchange reactions with other ions bound to the soil surface, chelation processes, and precipitation reactions) has generally been attributed to the reactions between metal ions and soil components, resulting in the transfer of metals from labile to non-available pools in the soil with time of ageing, which may take months to years to reach equilibrium (Hooda and Alloway, 1993; Ma et al., 2006; Wendling et al., 2009). In addition, it is well known that total metal concentrations poorly predict metal toxicity in the soil as only a fraction of the total metal content is bio(available) for the uptake and consequent adverse effects on soil organisms (Bradham et al., 2006; Lanno et al., 2004). Metal bioavailability and

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toxicity, therefore, differ between freshly spiked and aged (field) soils. Thus, ageing should be taken into account in the laboratory tests that are used for environmental risk assessment for better simulating the conditions of the natural terrestrial environment.

When determining metal toxicity in soil by adding highly soluble metal salts, counterions may also contribute in varying degrees to the observed toxic responses of soil organisms, through direct or indirect effects (Peredney and Williams, 2000; Schrader et al., 1998). For instance, some counterions (e.g. NO_3^- and Cl^-) were demonstrated to be toxic to nematodes (Peredney and Williams, 2000). Under field conditions, counterions could be removed through leaching by rainwater or by irrigation. Bongers et al. (2004) found that percolation of spiked LUFA 2.2 soil reduced the toxicity of Pb to the reproduction of *Folsomia candida*, and eliminated the difference in toxicity between $Pb(NO_3)_2$ and $PbCl_2$. Percolating the soils with deionized water or artificial or natural rain water before conducting ecotoxicity tests, therefore, was suggested as an efficient way to minimize the effects of the associated counterions in laboratory studies (Bongers et al., 2004; Smit and Van Gestel, 1998). Leaching metal-spiked soils did reduce metal toxicity in soils but could not fully explain the difference in metal toxicity between laboratory-spiked and field-contaminated soils, which could be explained by metal concentrations in pore water (Lock et al., 2006).

This study was designed to investigate the influence of leaching and metal form on Pb bioavailability and toxicity to the potworm *Enchytraeus crypticus* in freshly spiked and aged LUFA 2.2 soil. We aimed at: (1) assessing the effect of percolation on the sorption of Pb from different chemical compounds (PbO, $Pb(NO_3)_2$) freshly spiked into soil or after aging for 18 months, (2) comparing the toxicity of different chemical Pb forms (PbO, $Pb(NO_3)_2$), (3) investigating the effect of percolation on the toxicity of PbO and $Pb(NO_3)_2$ in freshly spiked and aged soils, and (4) determining which measurable Pb concentration could be the best expression of lead toxicity.

2. Materials and methods

2.1. Test organism

Enchytraeus crypticus (Enchytraeidae; Oligochaeta; Annelida) were cultured on agar prepared with an aqueous soil extract, in a climate room at 16 °C, with 75% relative humidity, and in complete darkness. The culture was maintained for more than 10 years in the Department of Ecological Science at Vrije Universiteit, Amsterdam. The animals were fed twice a week with a mixture of oat meal, dried yeast, yolk powder, and fish oil (Castro-Ferreira et al., 2012). Adult *E. crypticus* of approximately 1 cm with clearly visible clitella were used in the tests.

2.2. Test soils

Standard LUFA 2.2 soil was obtained from the LUFA Institute (Landwirtschaftliche Untersuchungs-und Forschungsanstalt) at Speyer, Germany. Soils were moistened to a final soil moisture content of 24% (w/w), which equals 50% of the maximum water-holding capacity (WHC). $Pb(NO_3)_2$ and PbO (purity >99.99%; Sigma-Aldrich; USA) were spiked as powder to the moist soil to obtain nominal concentrations of 0, 50, 100, 200, 400, 600, 800, 1600 and 3200 mg Pb/kg dry soil and 0, 78, 156, 312, 625, 1250, 2500, 5000 and 1000 mg Pb/kg dry soil, respectively. The spiked soils were incubated at 20 °C under stable climate conditions for 18 months (aged soils). Soil moisture content was frequently checked by weighing the boxes and moisture lost replenished by adding deionized water. After 18 months, a new batch of soil was freshly spiked with $Pb(NO_3)_2$ and PbO to obtain the same nominal Pb concentrations (freshly spiked soils), and equilibrated for 2 weeks before use. The leaching was performed as described by Bongers et al. (2004). Half of the freshly spiked and aged soils were percolated with an amount of deionized water equaling two times the amount of water contained in the moist soils. Afterwards, the leached soils were air dried to a final soil moisture content of 24% (w/w) and equilibrated for 1 week before use in the toxicity tests.

2.3. Toxicity tests

Effects of the two Pb forms on the survival and reproduction of *E. crypticus* in freshly spiked or aged soils with or without leaching were determined following OECD guideline 220 (OECD, 2016), modified by Castro-Ferreira et al. (2012). Mortality and reproduction were determined, and survivors were collected for internal Pb analysis after 21 d exposure in the freshly spiked and aged soils. Four replicates were used. For each replicate, ten adult worms were transferred to a 100 mL glass jar with 30 g moist soil and 2 mg oatmeal as food. Test jars were covered with perforated aluminum foil for air exchange and incubated at 20 °C, 75% relative humidity and a light/dark photoperiod cycle of 16/8 h. Once a week, food and moisture content of the soils were checked and replenished when necessary. After 21 d, survival was determined and three adults with clean guts from each replicate were stored at −20 °C for further analysis. For gut depuration, the animals were kept for 24 h in ISO solution containing 294 mg/L $CaCl_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 5.8 mg/L KCl and 64.8 mg/L $NaHCO_3$ (Sigma Aldrich, >99%) (ISO, 2012). Stained with Bengal rose (Sigma Aldrich, in 1% ethanol), the juveniles were counted on the pictures taken using Photoshop CS5.0.

2.4. Chemical analysis

Soils were dried at 40 °C for 48 h. To measure total Pb concentrations, about 130 mg dry soil samples were digested with 2 mL of a mixture of HNO_3 (65%, Sigma-Aldrich, USA) and HCl (37%, Sigma-Aldrich, USA) (4:1 v/v) in tightly closed Teflon containers and heated for 7 h at 140 °C in an oven (Binder FD). After cooling down, 8 mL deionized water was added to the container and Pb concentrations in the digests were measured by flame atomic absorption spectrometry (F-AAS; AAnalyst 100, Perkin Elmer, Germany). To check for the accuracy of the method, the certified reference material ISE sample 989 (Soil-Analytical Exchange, University of Wageningen, the Netherlands) was included in this study. Recoveries of Pb in the reference material were 91.0–98.4%. The detection limit for Pb analysis by F-AAS was 0.027 mg/L.

The frozen worms were freeze-dried for at least 24 h, weighted individually using an analytical balance (Mettler Toledo GmbH UMT2) and digested in 300 μL of a mixture of HNO_3 (65%; Mallbaker Ultrex Ultra-Pure) and $HClO_4$ (70%; Mallbaker Ultrex Ultra-Pure) (7:1 v/v) in a block heater (TCS Metallblock Thermostat) with a heating ramp ranging from 85 to 180 °C for 2 h. Afterwards, the Pb concentrations in the animal digests were determined by electrothermal atomization (ET) AAS with graphite furnace as an atomizer (PinAAcle 900Z, Perkin Elmer, Germany). The certified reference material DOLT 4 (Dogfish liver, LGC Standards) was used as the quality control of the analysis. The Pb recoveries were 93.3–101.5%. The detection limit for Pb in this analysis was 0.2367 μg/L.

Available Pb was measured as 0.01 M $CaCl_2$ extractable and porewater Pb concentrations. For $CaCl_2$-extraction (Ahnstrom and Parker, 1999; Bongers et al., 2004), 5 g dry soil samples were mixed with 25 mL 0.01 M $CaCl_2$ (Sigma Aldrich, >99%) solution (5:1, w/v) and shaken at 200 rpm for 2 h. The suspension was filtered over a cellulose nitrate membrane filter (0.45 μm) (Whatman) after settling overnight. For porewater (PW) extraction, 30 g dry soil was moistened to 100% WHC by adding deionized water and equilibrated for 1 week. The soil samples were filtered through a 0.45 μm cellulose nitrate membrane filter (Whatman) placed between 2 round paper filters (Whatman), and centrifuged at 2000 g at 15 °C for 45 min. Pb concentrations in the 0.01 M $CaCl_2$ extracts and pore water were measured by using flame or ET-AAS depending on the concentration level. Pore water was also analyzed for total Ca concentrations using F-AAS.

pH values in 0.01 M $CaCl_2$-extracts and pore water were determined using a pH meter (WTW, Inolab pH7110).

2.5. Data analysis

A Freundlich isotherm was used to describe the sorption of lead to the test soils. Pb uptake by the enchytraeids was related to soil Pb concentrations (total, 0.01 M $CaCl_2$-extractable and PW Pb concentrations), using a Langmuir isotherm. A logistic dose-response model was used to describe the relationship between enchytraeid survival or juvenile numbers and exposure concentrations (total, 0.01 M $CaCl_2$-extractable, and PW Pb concentrations in the soil, and body Pb concentrations in the animals). From this relationship lethal/effective concentrations causing 10% and 50% reduction in survival or in the number of juveniles produced (LC10, LC50, EC10 and EC50, respectively) and their corresponding 95% confidence intervals were estimated. A generalized likelihood-ratio test was used to compare sorption and toxicity data results for different treatments. To compare differences in parameters (like pH) between treatments or between ageing times, a student T test was applied.

All calculations used measured concentrations and were performed in SPSS 24.0.

3. Results

3.1. Soil analysis

The pH_{PW} and pH_{CaCl2} of control soils were 5.61 and 5.14 for aged soils and 5.93 and 5.65 for freshly spiked soils before leaching. For $Pb(NO_3)_2$, pH_{PW} and pH_{CaCl2} decreased with increasing total Pb concentration in the soil, while pH increased with increasing total Pb concentration for PbO (Figs. S1–S2). Percolation of the soil with deionized water slightly raised soil pH_{PW} for both Pb forms. Soil pH_{CaCl2} also showed the same trend which was not statistically significant.

The background Pb concentration in the control (LUFA 2.2 soil) was 15–22 mg Pb/kg dry soil. Measured total Pb concentrations in the freshly spiked and aged soils were in good agreement with the nominal levels. For both Pb forms, percolation only slightly affected the total soil Pb concentrations. In all treatments, the availability of Pb in the soil (determined as 0.01 M $CaCl_2$-extractable and PW Pb concentrations) was positively correlated with total Pb concentration for both Pb forms. Percolation did not affect Pb concentrations in $CaCl_2$-extracts in aged or freshly spiked soils for $Pb(NO_3)_2$, and slightly decreased $CaCl_2$-extractable Pb concentrations for PbO. After percolation, PW Pb concentrations were considerably lower in freshly spiked and aged soils for both Pb forms (Figs. S3–4). The changes in Pb availability were confirmed by Freundlich isotherms, which could well describe the sorption of Pb to the soils for both Pb forms, with $R^2 > 0.90$ (Table 1). The Freundlich adsorption constants (K_{FC}) based on $CaCl_2$-extractable Pb concentrations stayed constant at 787–791 and 1751–1797 $(L/kg)^n$ for $Pb(NO_3)_2$ in aged and freshly spiked soils after percolation, while they slightly increased from 4811 to 7355 $(L/kg)^n$ to 5395 and 9847 $(L/kg)^n$ for PbO, respectively. Percolation significantly affected K_{FP} based on Pb concentrations in pore water for both Pb forms ($\chi^2_{df=1} \geq 4.14$; $p < 0.05$), with K_{FP} increasing by a factor of 2.1–5.9 after percolation. Percolation significantly raised the corresponding n value from 0.510 to 0.519 to 0.941 and 0.854 for $Pb(NO_3)_2$ in aged and freshly spiked soils, respectively ($\chi^2_{df=1} \geq 23.2$; $p < 0.05$). And for PbO, they slightly increased after percolation.

In non-leached soils, Ca concentration in pore water was positively correlated with total Pb concentration in the soil for $Pb(NO_3)_2$, whereas it slightly decreased with increasing soil Pb concentration for PbO (Fig. S5). Percolation strongly reduced PW Ca concentrations to constant levels for both Pb forms in both freshly spiked and aged soils.

3.2. Pb bioaccumulation

Internal Pb concentrations of control animals from different treatments ranged from 0.49 to 2.99 mg Pb/kg dry body wt. Pb concentrations in the surviving worms exposed for 21 days in spiked soils increased with increasing exposure Pb concentration (total, $CaCl_2$-extractable and PW Pb concentrations) in the soil for both Pb forms and treatments. These relationships were well described by Langmuir models (Fig. 1). Based on total Pb concentrations in the soil, Pb uptake was higher in $Pb(NO_3)_2$-spiked soils than that in PbO-spiked soils, while there was no significant difference in internal Pb concentrations between non-leached and leached soils for both Pb forms in aged and freshly spiked soils ($\chi^2_{df=1} \leq 2.46$; n.s.). The differences between the two lead forms for all treatments almost disappeared when relating body Pb concentration to $CaCl_2$-extractable Pb concentrations in the soil. An overall Langmuir isotherm could well describe Pb accumulation in the enchytraeids from all treatments, with estimated maximum Pb uptake capacity of 89.5 mg Pb/kg dry body wt and $R^2 = 0.894$

Table 1
Parameters describing the sorption of Pb to freshly spiked and aged natural standard LUFA 2.2 soil with $Pb(NO_3)_2$ or PbO with or without percolation and related to 0.01 M $CaCl_2$ extractable and porewater Pb concentrations. Shown are the Freundlich sorption constant K_F ($(L/kg)^n$) (K_{FC} and K_{FP} based on $CaCl_2$ extractable and porewater Pb concentration, respectively) and slope parameter n with corresponding 95% confidence intervals.

Pb form	Treatment	Sorption related to $CaCl_2$-extractable Pb concentrations		Sorption related to porewater Pb concentrations	
		K_{FC}	n	K_{FP}	n
$Pb(NO_3)_2$	Aged	791 (735–847)	0.497 (0.472–0.522)	567 (465–647)	0.510 (0.458–0.593)
	Aged + leached	787 (676–898)	0.572 (0.516–0.628)	2155 (1307–2556)	0.941 (0.771–1.07)
	Freshly spiked	1751 (1463–2039)	0.506 (0.462–0.550)	1455 (1116–1743)	0.519 (0.453–0.585)
	Freshly spiked + leached	1797 (1612–1982)	0.499 (0.473–0.525)	5503 (3612–7395)	0.854 (0.754–0.955)
PbO	Aged	4811 (3678–5895)	1.07 (1.00–1.27)	10935 (7285–14586)	1.51 (1.40–1.75)
	Aged + leached	5395 (4372–6418)	1.10 (1.02–1.19)	22867 (6580–39149)	1.66 (1.34–1.98)
	Freshly spiked	7355 (5589–9122)	0.747 (0.679–0.814)	64121 (37494–90760)	1.55 (1.41–1.69)
	Freshly spiked + leached	9847 (7746–11947)	0.794 (0.736–0.852)	376877 (114528–639297)	1.86 (1.66–2.07)

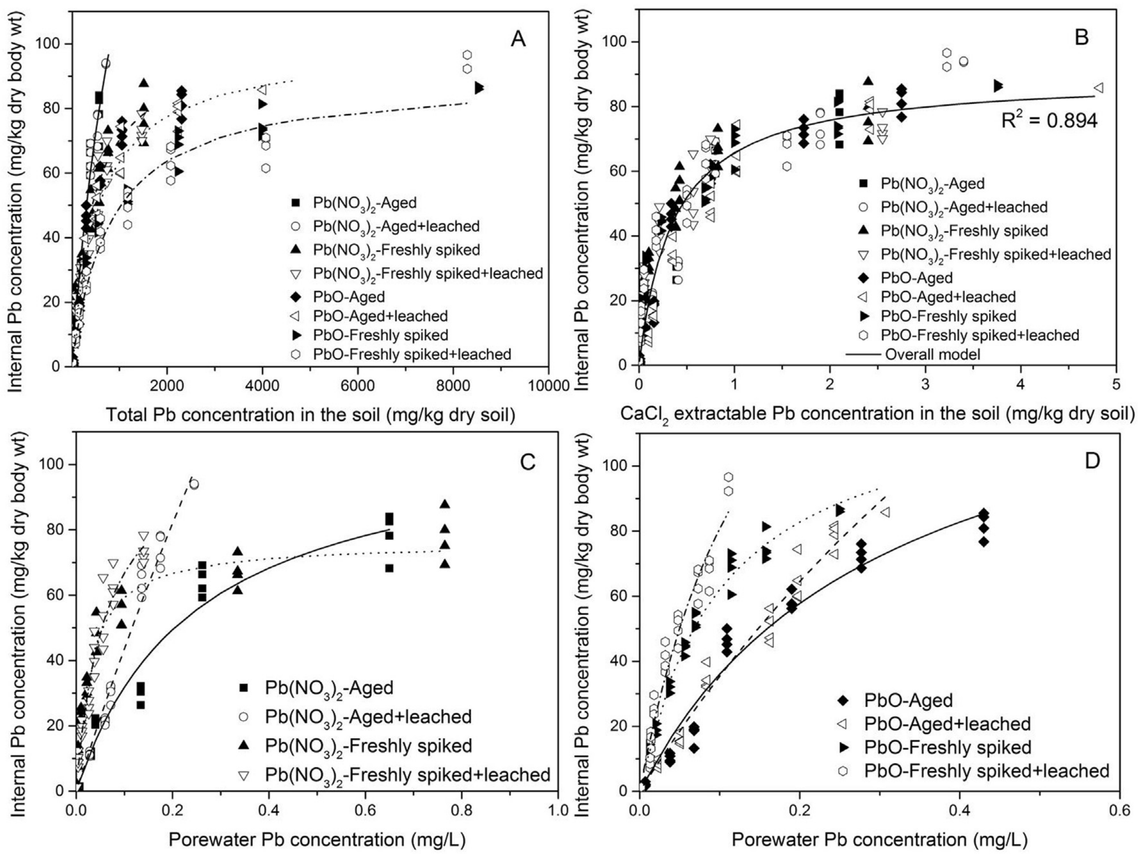

Fig. 1. Lead concentrations in surviving adult *Enchytraeus crypticus* after 3 weeks exposure in freshly spiked and aged natural LUFA 2.2 soil spiked with $Pb(NO_3)_2$ or PbO with or without percolation, related to total Pb concentration in soil (A), 0.01 M $CaCl_2$-extractable Pb concentrations in soil (B) and Pb concentrations in pore water (C and D). Dots represent measured concentrations, lines show the fit of a Langmuir isotherm to the data.

(Fig. 1B). Related to PW Pb concentrations, Pb uptake in the animals slightly increased after percolation for both Pb forms in both freshly and aged spiked soils.

3.3. Pb toxicity

The control performance of the enchytraeids satisfied the validity criteria of the test guideline (OECD, 2016), with mean adult survival being > 95%, and on average 740–878 juveniles per test jar. The dose-response curves for the effects of $Pb(NO_3)_2$ and PbO on the survival and reproduction of *E. crypticus* after 21 d of exposure in freshly spiked and aged soils with or without percolation are shown in Figs. 2 and 3. The estimated LC50, LC10, EC50, and EC10 values, expressed on the basis of total and 0.01 M $CaCl_2$-extractable Pb concentrations in soil, Pb concentrations in pore water and internal Pb concentrations in the surviving adults, are summarized in Table 2. Percolation slightly raised LC50s based on total soil Pb concentrations for $Pb(NO_3)_2$ from 530 to 1380 mg Pb/kg dry soil to 608 and 1538 mg Pb/kg dry soil in aged and freshly spiked soils, respectively, while it did not affect those for PbO being 1881–1889 mg Pb/kg dry soil and 5614–5815 mg Pb/kg dry soil. EC50s based on total Pb concentrations increased from 129 to 394 mg Pb/kg dry soil to 258 and 601 mg Pb/kg dry soil for $Pb(NO_3)_2$ and PbO in freshly spiked soils, respectively. In aged soils, EC50s remained similar at 99.4–103 mg Pb/kg dry soil for $Pb(NO_3)_2$ but for PbO they slightly increased from 97.5 to 124 mg Pb/kg dry soil after percolation.

LC50s on the basis of 0.01 M $CaCl_2$-extractable Pb concentrations ranged from 1.72 to 2.78 mg Pb/kg dry soil and EC50s from 0.044 to 0.173 mg Pb/kg dry soil for both Pb forms from different treatments. Pb concentrations in $CaCl_2$-extracts could well explain the effects of both Pb forms and percolation, as confirmed by one overall logistic dose-response curve fitting well to all the data on survival and reproduction, with $R^2 = 0.953$ and 0.954, respectively (Figs. 2C and 3C). When based on PW Pb concentrations, LC50s ranged from 0.097 to 0.686 mg/L and EC50s from 0.012 to 0.063 mg/L for both Pb forms from different treatments. Pb uptake in the enchytraeids could well predict the survival of adults for both Pb forms from different treatments with a single overall dose-response curve ($R^2 = 0.791$) (Fig. 2F). The estimated LC50s based on internal Pb concentrations were 76.4–84.5 mg Pb/kg dry body wt. Pb effects on reproduction however, could not be described by body Pb concentration in the enchytraeids for both Pb forms in the freshly spiked and aged soils with and without percolation. EC50s based on body Pb concentration ranged from 12.0 to 41.1 mg Pb/kg dry body wt.

4. Discussion

The novelty of this study is in the assessment of the effect of percolation on the (bio)availability of different Pb forms ($Pb(NO_3)_2$ and PbO) to *E. crypticus* exposed in a natural standard soil freshly spiked or aged for 18 months. Percolation reduced PW Pb concentrations and Pb toxicity for both Pb form in both the freshly spiked and aged soils. The influence of percolation differed between the two Pb forms as well as between freshly spiked and aged soils.

The differences of Pb availability ($CaCl_2$-extractable and PW Pb concentrations) between freshly spiked soils and aged soils were

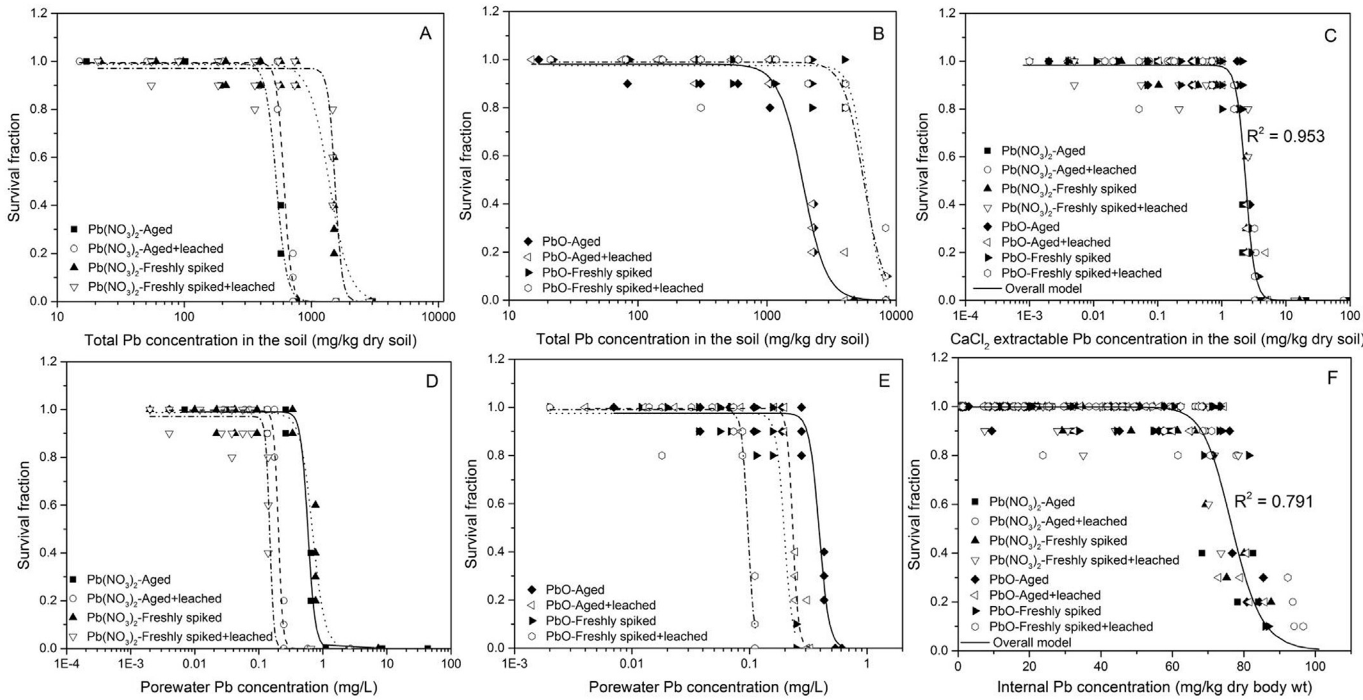

Fig. 2. Effects of $Pb(NO_3)_2$ or PbO on the survival of *Enchytraeus crypticus* after three weeks exposure in freshly spiked and aged natural LUFA 2.2 soil with or without percolation. Pb concentrations are expressed as total (A and B) and 0.01 M $CaCl_2$ extractable concentrations in soil (C), concentrations in pore water (D and E), or internal concentrations in surviving adults (F). Lines show the fit of a logistic dose-response curve; in cases where only one curve is shown, the dose-response curves for the different treatments and Pb forms did not significantly differ.

mainly due to the use of a different batch of LUFA 2.2 soil with slightly different soil properties for the tests on freshly spiked soil (e.g. soil pH shown in Figs. S1–S2 and PW Ca shown in Fig. S5). Much higher Pb availability ($CaCl_2$-extractable and PW Pb concentration) was found in $Pb(NO_3)_2$-spiked soils than in PbO-spiked soils, as shown by the large differences in the partition coefficients K_{FC} and K_{FP}. This might due to the huge difference in solubility of these two Pb forms. Being a highly soluble Pb form, $Pb(NO_3)_2$ could release more Pb into the soil solution while the dissolution of PbO might have taken months to years to reach equilibrium. Elless and Blaylock (2000) observed similar results with 39% of total Pb in the soil being extracted with 1 M $MgCl_2$ for $Pb(NO_3)_2$, whereas there was only 20% exchangeable Pb for PbO in soil spiked at 600 mg Pb/kg dry soil. Thus, the chemical form may have great influence on Pb availability in soil.

Percolation with deionized water did not change the $CaCl_2$-extractable Pb concentration for both Pb forms tested. This was partly in agreement with the findings of Bongers et al. (2004), who observed no significant differences in Pb availability, determined by water and 0.01 M $CaCl_2$ extraction, between non-leached and leached soils. Also no significant differences were found in $CaCl_2$-extractable Zn concentrations, while water-extractable Zn concentrations declined after percolation in 2-months aged OECD artificial soil (Lock and Janssen, 2002). In the present study, percolation dramatically decreased PW Pb concentrations for both Pb forms in freshly spiked and aged soils, which agreed with the results of Lock et al. (2006). This leads us to conclude that metal concentrations in the soil solution may decrease after percolation, without leading to differences in metal concentrations obtained by applying stronger extractants (e.g. 0.01 M $CaCl_2$, 1 M $MgCl_2$ or 1 M ammonium nitrate). Thus leaching only removed the metal fraction readily available in the soil solution and did not affect other metal fractions.

The influence of percolation on PW Pb concentrations differed between the two Pb forms and between freshly spiked and aged soils. Pb concentrations in pore water were reduced by a factor of 0.94–2.25 after percolation for PbO, but up to a factor of 16.0 for $Pb(NO_3)_2$. As its solubility is the primary factor determining the transport of a metal in soil, PW Pb concentrations were much higher in $Pb(NO_3)_2$-spiked soils than in PbO-spiked soils. But PW Pb could partly be replenished by the dissolution (release) of PbO. Thus, PW Pb was more affected by percolation in $Pb(NO_3)_2$-spiked soils than in PbO-spiked soils. Percolation increased K_{FP} values by factors of 3.9–5.9 and 2.1–3.9 in freshly spiked and aged soils, respectively, confirming the stronger influence of percolation in freshly spiked soils than in aged soils. This might be due to the insufficient replenishment by desorption of Pb in the soil as the fraction of metal that is reversibly bound can decrease during ageing (Stevens et al., 2003; Lock et al., 2006).

In both freshly spiked and aged soils and for both Pb forms percolation significantly reduced the concentrations of PW Ca to the levels found in the control soils. Similar results were observed by Smit and Van Gestel (1998), who found lower water-extractable Ca concentrations in leached soils than in non-leached soils and independent of total Zn concentration in the soil. These findings strongly suggest that only a small fraction of the PW Ca is strongly bound and therefore not removed by leaching with deionized water (Kinraide and Yermiyahu, 2007; Milne et al., 2003). Similar results were observed in Ni-spiked soil, where soil solution Ca concentrations strongly decreased after leaching (Oorts et al., 2006).

After 21 days of exposure, the accumulation of Pb in the enchytraeids was positively correlated with total, $CaCl_2$-extractable and PW Pb concentrations in the soil for both Pb forms from different treatments. The Pb concentrations in *E. crypticus* were comparable with those found in other studies with exposure to

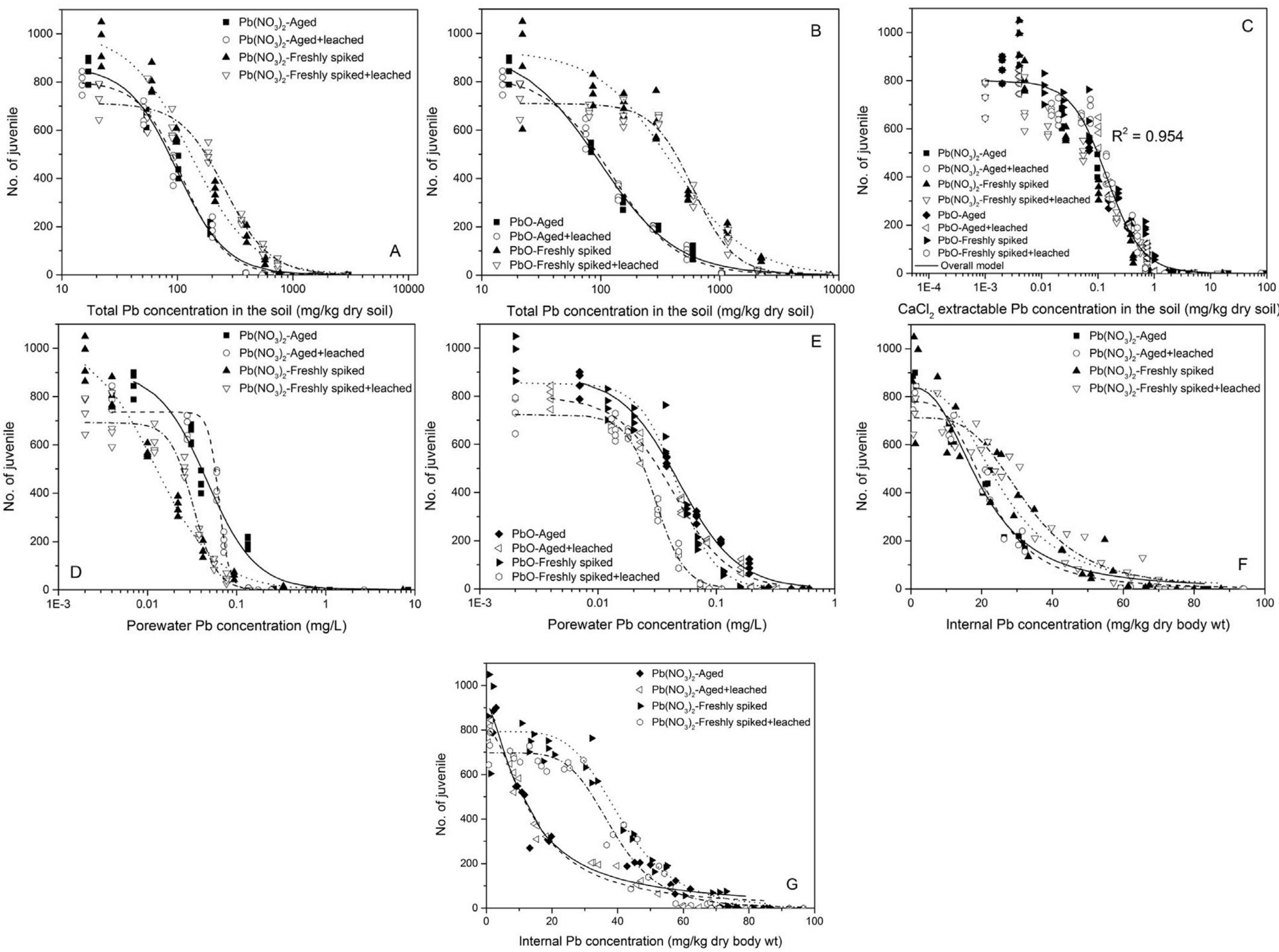

Fig. 3. Effects of $Pb(NO_3)_2$ or PbO on the reproduction of *Enchytraeus crypticus* after three weeks exposure in freshly spiked and aged natural LUFA 2.2 soil with or without percolation. Pb concentrations are expressed as total (A and B) and 0.01 M $CaCl_2$ extractable concentrations in soil (C), concentrations in pore water (D and E) or internal concentrations in the surviving adults (F and G). Lines show the fit of a logistic dose-response curve; in cases where only one curve is shown, the dose-response curves for the different treatments and Pb forms did not significantly differ.

$Pb(NO_3)_2$- and $PbCl_2$-amended LUFA 2.2 soil (Zhang and Van Gestel, 2017). Metal form (solubility) was demonstrated to have great impacts on the metal uptake by soil organisms (Davies et al., 2003; Romero-Freire et al., 2017). Due to the difference in solubility of the two Pb forms, *E. crypticus*, therefore, accumulated more Pb when exposed to $Pb(NO_3)_2$ compared to PbO in this study.

Although percolation reduced PW Pb concentrations by a factor 4.5, it did not significantly affect Pb uptake in the surviving worms for both Pb forms. There are two possible explanations for this observation. First, pore water is the major route for metal uptake by soil invertebrates (Van Gestel, 2012) and according to the Biotic Ligand Model (BLM) theory, the decrease of PW Ca^{2+} and H^+ concentrations after percolation might reduce competition with Pb^{2+} for effective binding sites on the surface of the target organisms. Thus, the percolation-independent internal Pb concentration can be attributed to the fact that reduced levels of Pb went along with a reduction of the competing ions (e.g. Ca^{2+} and H^+) in the soil solution. PW Pb concentration itself, therefore, could not well predict Pb accumulation in the enchytraeids. Another explanation is that the 0.01 M $CaCl_2$-extractable fraction of Pb in the soil represents the actual bioavailable pool for Pb accumulation in the enchytraeids (Kim et al., 2015). As mentioned above, Pb concentrations in the $CaCl_2$ extracts did not differ in non-leached and leached soils, leading to similar internal Pb concentrations. Thus, relating to $CaCl_2$-extractable Pb concentrations minimized the difference in internal Pb concentrations in *E. crypticus* between the two Pb forms and between non-leached and leached soils.

The toxicity values (LC50s and LC10s) based on total Pb concentration in the soil slightly increased after percolation for $Pb(NO_3)_2$, but did not differ between non-leached and leached soils for PbO. The reduction in the toxicity of $Pb(NO_3)_2$ is consistent with the findings of Bongers et al. (2004), who observed up to 2.2-fold reduction in Pb toxicity to the survival of *Folsomia candida* by leaching $Pb(NO_3)_2$-spiked soils with deionized water, while no such effect was seen for $PbCl_2$-spiked soils. After spiking with dissolved metal salts, soils show increased soluble salt contents in the soil solution, relative to the amount of metal added, referred to as the "salt effect", which might have direct or indirect adverse effects on soil organisms (Peredney and Williams, 2000; Schrader et al., 1998). Leaching was found to be a useful method to avoid the contribution of the associated counterions on metal toxicity (Bongers et al., 2004; Smit and Van Gestel, 1998). Thus, the decrease of LC50s based on total Pb concentrations for $Pb(NO_3)_2$ in freshly spiked and aged soils could partly be attributed to the decrease of the concentration of the counterion (salinity) due to leaching effects. This also is supported by the loss of Ca from the pore water. The absence

Table 2
LC50, LC10, EC50 and EC10 values (with 95% confidence intervals) for the effects of $Pb(NO_3)_2$ or PbO on the survival and reproduction of *Enchytraeus crypticus* exposed for 21 days in freshly spiked and aged natural standard LUFA 2.2 soil with or without percolation. Effect concentrations are expressed on the basis of total and 0.01 M $CaCl_2$ extractable Pb concentrations in soil, Pb concentrations in pore water and Pb concentrations measured in surviving animals.

	Treatment	$Pb(NO_3)_2$				PbO			
		Total Pb (mg/kg dry soil)	$CaCl_2$ Extractable Pb (mg/kg dry soil)	Porewater Pb (mg/L)	Internal Pb (mg/kg body wt)	Total Pb (mg/kg dry soil)	$CaCl_2$ Extractable Pb (mg/kg dry soil)	Porewater Pb (mg/L)	Internal Pb (mg/kg body wt)
LC50	Aged	530 (513–547)	1.72 (1.59–1.86)	0.583 (0.495–0.672)	76.8 (74.9–78.8)	1889 (1753–2025)	2.45 (2.34–2.55)	0.391 (0.373–0.410)	78.7 (77.2–80.3)
	Aged + leached	608 (594–622)	2.42 (2.30–2.54)	0.201 (0.195–0.207)	84.4 (83.1–85.7)	1881 (1766–1995)	2.01 (1.87–2.15)	0.233 (0.229–0.237)	76.4 (73.7–79.1)
	Freshly spiked	1380 (1292–1469)	2.07 (1.86–2.28)	0.686 (0.633–0.739)	77.3 (74.6–80.0)	5815 (5136–6495)	2.79 (2.53–3.05)	0.197 (0.184–0.211)	83.3 (82.6–84.1)
	Freshly spiked + leached	1538 (–)	2.78 (–)	0.148 (–)	83.6 (71.6–97.2)	5614 (5190–6037)	2.16 (1.99–2.33)	0.097 (0.095–0.100)	84.5 (81.5–87.4)
LC10	Aged	431 (391–471)	1.03 (0.799–1.27)	0.438 (0.201–0.675)	68.2 (64.5–72.0)	1191 (970–1413)	1.87 (1.66–2.08)	0.317 (0.277–0.358)	71.9 (69.1–74.8)
	Aged + leached	527 (512–542)	1.79 (1.68–1.90)	0.169 (0.163–0.175)	73.1 (71.3–74.9)	1183 (990–1376)	1.22 (0.989–1.45)	0.206 (0.196–0.216)	64.6 (57.8–71.3)
	Freshly spiked	930 (710–1150)	1.08 (0.672–1.48)	0.421 (0.295–0.547)	65.4 (60.8–70.0)	4357 (3705–5009)	2.23 (1.97–2.50)	0.165 (0.151–0.181)	80.5 (79.4–81.6)
	Freshly spiked + leached	1336 (–)	2.13 (–)	0.126 (–)	64.5 (54.8–73.2)	3789 (3392–4186)	1.44 (1.29–1.60)	0.085 (0.082–0.088)	70.6 (66.4–74.7)
EC50	Aged	99.4 (91.0–108)	0.093 (0.069–0.117)	0.046 (0.040–0.053)	20.1 (18.2–22.0)	97.5 (83.9–111)	0.101 (0.091–0.112)	0.048 (0.044–0.052)	12.0 (8.9–15.1)
	Aged + leached	103 (94.1–113)	0.173 (0.153–0.192)	0.063 (0.061–0.065)	22.1 (20.5–23.7)	124 (112–136)	0.160 (0.142–0.179)	0.043 (0.039–0.047)	14.5 (12.7–16.3)
	Freshly spiked	129 (111–146)	0.044 (0.025–0.062)	0.012 (0.009–0.014)	25.5 (21.3–29.7)	394 (323–465)	0.123 (0.081–0.164)	0.047 (0.043–0.052)	41.1 (38.0–44.2)
	Freshly spiked + leached	258 (229–287)	0.109 (0.084–0.134)	0.033 (0.030–0.035)	32.7 (29.8–35.7)	601 (547–655)	0.168 (0.143–0.193)	0.031 (0.029–0.032)	38.6 (36.0–41.1)
EC10	Aged	33.9 (27.1–40.9)	0.011 (0.005–0.017)	0.011 (0.008–0.015)	8.5 (6.55–10.4)	18.8 (12.7–24.9)	0.015 (0.011–0.019)	0.014 (0.011–0.016)	2.7 (1.2–4.2)
	Aged + leached	40.5 (36.8–44.4)	0.048 (0.036–0.059)	0.047 (0.043–0.052)	11.3 (9.1–13.4)	30.7 (23.6–38.1)	0.041 (0.030–0.053)	0.013 (0.010–0.016)	4.2 (3.1–5.4)
	Freshly spiked	33.4 (23.5–43.0)	0.004 (0.001–0.008)	0.002 (0.001–0.003)	11.9 (7.3–16.6)	79.8 (46.3–113)	0.013 (0.003–0.022)	0.021 (0.016–0.027)	26.5 (21.7–31.2)
	Freshly spiked + leached	97.5 (72.9–112)	0.016 (0.008–0.024)	0.017 (0.014–0.020)	18.1 (14.5–21.6)	253 (200–305)	0.045 (0.029–0.061)	0.017 (0.014–0.018)	25.8 (21.8–29.8)

- Data did not allow calculating 95% confidence intervals.

of an effect of percolation on the LC50s for PbO might be due to the absence of an associated counterion contributing to toxicity. This shows that the effect of percolation on Pb toxicity due to removal of the counterion is dependent on the chemical form added to the soil. Compared to mortality, reproduction is a more sensitive endpoint to evaluate Pb toxicity for *E. crypticus*. In this study, percolation increased EC50s based on total Pb concentrations to enchytraeid reproduction for $Pb(NO_3)_2$ and PbO in freshly spiked soils only by a factor of 2.0 and 1.5, respectively. Similar results were found by Bongers et al., who observed 1.3 and 2.9-fold reductions in Pb toxicity to collembolan (*F. candida*) reproduction by percolation of $PbCl_2$—and $Pb(NO_3)_2$-spiked soils, respectively. Lock et al. (2006), however, documented no difference in Pb toxicity to the reproduction of *F. candida* exposed to soils freshly spiked with $Pb(NO_3)_2$ compared to soils leached with synthetic rainwater. The observed difference in the influence of percolation might be partly due to the different leaching procedures and the use of test soils with different properties. Only few studies on the effects of percolation on Pb toxicity in PbO-spiked soils or aged soils could be found in the literature. Percolation did not affect Pb toxicity to potworm reproduction for both Pb forms in aged soils, suggesting that ageing might have impacts on the effects of percolation through influencing the metal exchangeable fraction in soils (Huang et al., 2015).

Estimating toxicity based on available concentrations in the soil often provides much better predictions compared to total metal concentrations (Bradham et al., 2006; Zhang and Van Gestel, 2017). In this study, LC50s and EC50s based on total Pb concentrations varied between the two Pb forms from different treatments. The effects on survival and reproduction of the worms were best explained from $CaCl_2$-extractable Pb concentrations in the soil ($R^2 > 0.953$). Since mortality only occurs when the body metal concentration reaches a certain level (lethal body concentration) where the capacity of detoxification mechanism is exceeded (Jager et al., 2011; Vijver et al., 2004), also a good relationship between survival of *E. crypticus* and internal Pb concentrations was found, showing that also metal bioaccumulation might be a good indicator for metal toxicity in the soil.

5. Conclusion

Percolation did not affect the total and $CaCl_2$-extractable Pb concentrations in the soil, while it did considerably reduce Pb concentrations in pore water for $Pb(NO_3)_2$ and PbO. No significant differences in Pb bioaccumulation in *E. crypticus* were found between non-leached and leached soils for both Pb forms. $Pb(NO_3)_2$ was more toxic to *E. crypticus* than PbO in both freshly spiked and 18-months aged soils. Percolating the soils before ecotoxicological tests slightly reduced the toxicity of $Pb(NO_3)_2$ for lethal effects, whereas it did not affect the toxicity of PbO. For the sublethal effects on reproduction, percolation had a greater influence in freshly spiked soils than in aged soils. Generally, the effect of percolation on the toxicity of Pb-spiked soils was dependent on the chemical form used for spiking as well as on ageing. PW Pb concentration could not well explain Pb toxicity, but body Pb concentrations may be a good indicator of Pb toxicity to survival of enchytraeids. When expressed on the basis of 0.01 M $CaCl_2$-extractable Pb concentrations, the difference in toxicity between Pb forms and treatments almost disappeared, showing that $CaCl_2$-extractable Pb was the best indicator of Pb toxicity in the soils.

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Appendix A. Supplementary data

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